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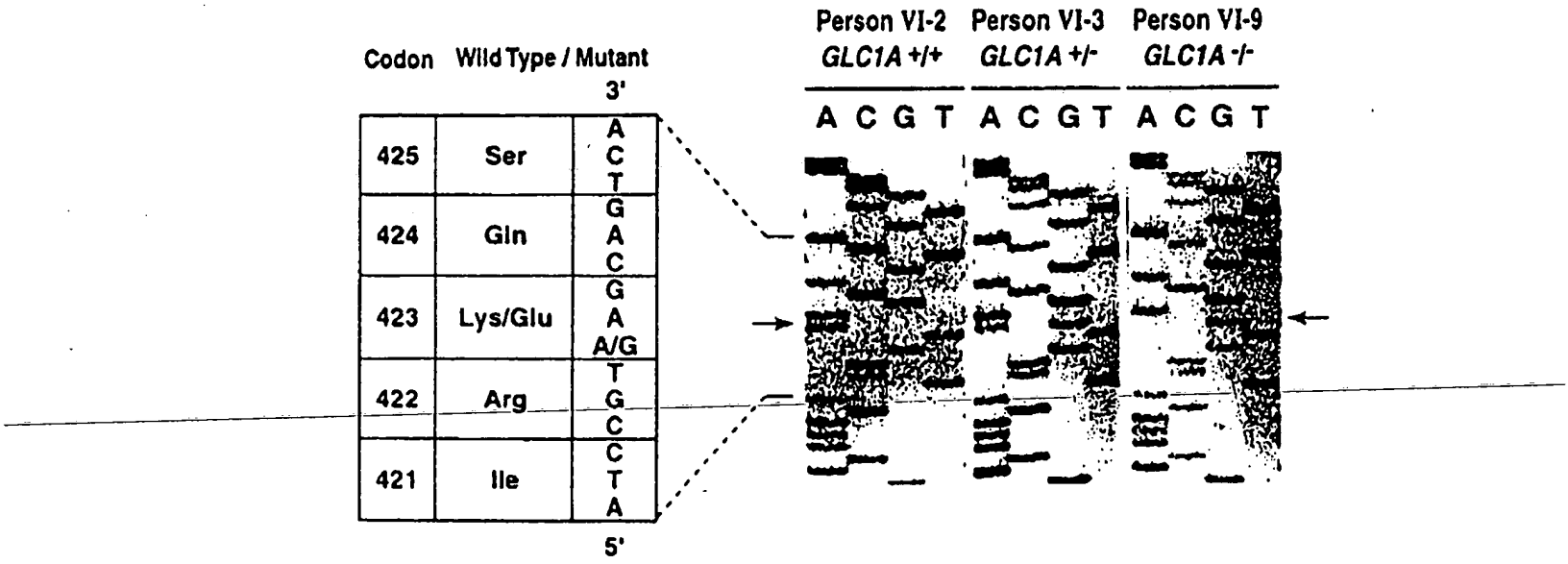
(54) **DIAGNOSTIC MOLECULAIRE DES GLAUCOMES ASSOCIES AUX CHROMOSOMES 1**

(54) **MOLECULAR DIAGNOSTIC OF GLAUCOMAS ASSOCIATED WITH CHROMOSOMES 1**



(57) On dévoile que, dans une maladie héréditaire autosomique, un mutant homozygote a un phénotype normal, ainsi que les utilisations que l'on peut faire de cette découverte. Un moyen facile et efficace de détecter des mutations dans le gène GLC1A/TIGR est également divulgué.

(57) The present invention discloses the discovery that in an autosomally inherited disease, a homozygote mutant is found to be phenotypically normal and the uses of such a knowledge thereof. The present invention has designed an easy and efficient means to detect mutations in the GLC1A/TIGR gene.

TITLE OF THE INVENTION

MOLECULAR DIAGNOSTIC OF GLAUCOMAS ASSOCIATED WITH CHROMOSOME 1

FIELD OF THE INVENTION

The present invention relates to the identification of mutations in the *GLC1A* gene and the detection of these mutations in individuals. The invention also relates to individuals being genotypically homozygote mutant in an autosomal dominant inherited disease yet being phenotypically normal.

BACKGROUND OF THE INVENTION

Glaucoma encompasses a complex of ocular-disease entities characterized by an optic neuropathy in which degeneration of retinal ganglion cells leads to a characteristic excavation of the head of the optic nerve (Shields et al., 1996, The Glaucomas, 2:717-725). Such damage causes progressive narrowing of the visual fields and, when uncontrolled, blindness. Affected people often have ocular hypertension defined as intraocular pressures consistently >21 mm Hg in both eyes. Although ocular hypertension is no longer an obligatory diagnostic criterion for glaucoma, it is still recognized as one of the most important risk factors (Wilson et al., 1996, The Glaucomas, 2:753-763). Until now, a diagnosis of glaucoma is made after observation of the characteristic atrophy of the optic nerve, which is associated with typical visual field defects.

In 1992, the World Health Organization estimated that, in the global population, 5.2 million people were blind as a result of

glaucoma (Thylefors et al., 1994, World Health Organ. Bull., 72:323-326), making it the third leading cause of blindness worldwide. The most common form is adult-onset primary open-angle glaucoma (MIM 137760; McKusick, 1994, Johns Hopkins University Press, p. 272), which represents ~50% of all cases of glaucoma. Among Caucasians, this form of the disorder affects ~2% of the population >45 years old (Leske, 1983, Am. Epidemiol., 118:166-191; Thylefors et al., 1994, supra; Wilson et al., 1996, supra). In African Americans, prevalence of adult-onset open-angle glaucoma is three to four times higher than that observed in White Americans. More than 15 million North Americans may have some form of glaucoma, but at least half of them may not be aware of it.

The glaucomas traditionally have been grouped into three categories: open angle, closed angle (also termed "angle closure"), and congenital. Each subtype has been further arbitrarily subdivided into *primary*, when the anterior chamber of the eye appears normal and no cause for glaucoma can be identified, or *secondary*, when glaucomas are caused by underlying ocular or systemic conditions (Shields et al., 1996, supra). Whereas the division between open and closed angles refers to the configuration of the irido-corneal angle in the anterior chamber of the eye, congenital glaucoma is used to define one of the many types of developmental glaucoma that usually occurs within the 1st year of life. The majority (60%-70%) of primary glaucomas are of the open-angle type. Primary open-angle glaucomas have been further subdivided into two groups according to age at onset, severity, and mode of inheritance: the more pr valent is middle- to late-age-onset chronic open-angle glaucoma (COAG), by convention diagnosed after age 35 years and characterized by its slow, insidious course (Shields et al., 1996, supra;

Wilson et al., 1996, supra). The less common form, juvenile open-angle glaucoma (JOAG), occurs between 3 years of age and early adulthood and generally manifests highly elevated intraocular pressures with no angle abnormalities (Goldwyn et al., 1970, Arch. Ophtalmol., 84:579-582; François, 1980, Am. J. Ophtalmol., 3:429-449; Johnson et al., 1996*a*, The Glaucomas, 1:39-54).

Although the precise molecular defects leading to open-angle glaucomas remain partly unknown, numerous advances in basic and clinical sciences have begun to identify the molecular basis of glaucomas by mapping the gene loci involved in the disease process. Due to recent mapping successes, the different forms of glaucoma will be further identified by the names of the loci to which they have been localized. According to the Human Genome Organization/Genome Database nomenclature, "*GLC*" is the general symbol for the glaucoma genes; "1", "2", and "3" are, respectively, the symbols for the open-angle, angle-closure, and congenital subtypes of glaucoma; and "A", "B", and "C" refer, respectively, to the first, second, or third gene mapped in each subgroup.

JOAG is a rare but aggressive form of glaucoma that usually segregates in an autosomal dominant fashion with high penetrance (Stokes, 1940, Arch. Ophthalmol., 24:885-909; Crombie et al., 1964, Br. J. Ophtalmol., 48:143-147; Lee et al., 1985, Ann. Ophtalmol., 17:739-741; Johnson et al., 1993, Ophthalmology, 100:524-529). In a single large American pedigree affected by an autosomal dominant form of JOAG, Sheffield et al. (1993, Nat. Genet., 4:47-50) located a gene responsible for this condition, at 1q21-q31. This locus, being the first open-angle glaucoma locus to be mapped, was

named "*GLC1A*." The *GLC1A* disease gene consistently was associated with onset of the JOAG phenotype before the age of 70 years, highly elevated intraocular pressures, and typical excavation of the head of the optic nerve. Gonioscopy showed open angles with no anterior-chamber abnormalities. The *GLC1A* has subsequently been reported by Nguyen et al. in US Patent 5,606,043 to encode the trabecular meshwork induced glucocorticoid response (*TIGR*) gene. The gene sequence was first submitted (13-JAN-1997) by Nguyen et al. to the GeneBank accession # U85257. The *TIGR* sequence was modified on 19 April 1997 in GeneBank following modifications by Nguyen submitted on 02-APR-1997. The accession number stayed the same # U85257.

Genetic maps of the human genome can be exploited to rapidly locate human monogenic disorders. The final version of the Généthon linkage map, which spans close to 100 % of the human genome, was published in March 1996 (Dib et al., 1996, Nature, 380:152-154). This map consists of 5,264 short tandem (AC/TG)n repeat polymorphisms with a mean heterozygosity of 70%.

The nomenclature system for the markers is well known in the field. The nomenclature used is decided by the Human Genome Organization (HUGO) nomenclature committee. It is as follows: for anonymous DNA sequences, the convention is to use D which is equivalent to DNA followed by 1-22, X or Y to denote the chromosomal number and location, then S stands for a unique segment and finally a serial number. For example, marker D2S2161 is a DNA marker located on chromosome 2 representing a unique segment. Its serial number is 2161.

The nomenclature for the glaucoma genes is the following:

"*GLC*" is the general symbol for the glaucoma genes; "1", "2", and "3" are, respectively, the symbols for the open-angle, angle-closure, and congenital subtypes of glaucoma; and, "A", "B" and "C" refer, respectively, to the first, second, or third gene mapped in each subgroup. For example, the *GLC1A* locus was the first open-angle glaucoma locus to be mapped, in this case to chromosome 1q23-q25 in 1993. It was later identified as the trabecular meshwork inducible glucocorticoid response gene product (*TIGR*) (Stone et al, 1997, Science, 275: 668-670).

These markers are accessible to all individuals. The central data resource for the human gene mapping effort is the Genome Data Base (GDB). It was established at Johns Hopkins University, School of Medicine. GDB is updated regularly. It collects, organizes, stores and distributes human genome mapping information. GDB is accessible electronically at WWW-URL: http://gdbwww.gdb.org/.

Alternatively, all the markers disclosed herein, except D6S967, are short (CA)n repeat markers that have been developed in the Généthon laboratory near Paris, France. These markers are also accessible electronically at WWW-URL: http://www.genethon.fr/.

Therefore markers are accessible either at GDB or at Généthon.

The first mutations identified in the *TIGR* gene that have been shown to give rise to glaucoma were first reported by Stone et. al (Science, 1997, 275:668-670). There are three mutations report d. No other mutations relating to the *TIGR* gene have been reported. The methodology used to identify these mutations was by amplifying

overlapping regions by polymerase chain reaction (PCR), performing single-strand conformational polymorphism (SSCP) on the amplification products and sequencing those DNA products that produced aberrant band pattern on the SSCP. No quick method for mutational analyses for the *TIGR* has been proposed.

The prior art as a whole teaches that a homozygote mutant for an autosomal dominant disease should display a higher penetrance than a heterozygote mutant. Heterozygote for an autosomal dominant disease often exhibits variable penetrance.

The present description refers to a number of documents the content of which is herein incorporated by reference.

SUMMARY OF THE INVENTION

The invention concerns the mutational analyses in the *GLC1A* gene locus encoding the *TIGR* gene (GeneBank accession no. U85257).

The present invention provides means to identify at least two nucleotide changes in the DNA sequence coding for *TIGR* that result in an amino acid change in the *TIGR* gene.

The invention further demonstrates that these amino acid changes result in mutations producing a disease state in individuals, the disease being glaucoma.

The early detection of individuals at risk for developing glaucoma is an important aspect of this invention. Early detection allows for intervention prior to the genesis of the disease process and disease progression and may obviate the symptoms and th onset of the disease.

A method for mutation analyses call d amplification

refractory mutation system (ARMS), that is simple and quick is disclosed herein. The proposed invention relates to the inclusion of primers and probes for the amplification and detection of all mutations in the *TIGR* gene. The invention teaches the use of the method of ARMS as related to glaucoma but the invention is not limited to this method for mutation analyses. Other methods known in the field for mutation analyses such as allele specific oligonucleotide (ASO), denaturing gradient gel electrophoresis (DGGE) and artificially created restriction site (ACRS) can also be used.

These mutation detection and analyses can be performed on either genomic DNA or DNA that has been transcribed to cDNA by any method known to a person skilled in the art.

In addition the applicant has demonstrated for the first time a new type of dominance in mammals in which heterozygotes have a much higher penetrance rate for a disease gene mutation than their homozygotic counterparts.

Further it is provided for the first time in an autosomal dominant disease that a homozygote mutant is phenotypically normal. Even though such an individual may give rise to an affected heterozygote offspring.

The invention provides applications and uses for such a discovery. These include but are not limited to:

a) treatment of heterozygote mutant affected individuals with overexpressed mutant protein to induce protein complementation such that normal protein function can be restored, this application will apply to any autosomal dominant disease exhibiting the same mod of action as describ d herein.

b) similarly an individual being a heterozygote for an autosomal dominant disorder exhibiting the same mode of action as described herein can be treated by gene therapy, such that a mutant allele is inserted into a vector and delivered to an individual thereby negating the effect of the heterozygote mutation by either allelic or protein complementation.

c) with this new knowledge a transgenic animal designed to carry a deleterious autosomal dominant mutation can be used to assess the requirement to produce a phenotypically normal animal, by either allelic complementation or protein complementation.

d) a diagnostic means to identify phenotypically normal genotypically mutant individuals that can transmit the mutant allele to their offsprings.

e) this knowledge can be used for showing dimerisation of *TIGR* peptides.

In accordance with the present invention there is therefore provided the means to easily identify novel mutations in the *TIGR* gene, wherein these mutations give rise to glaucoma. These mutations can also be identified by any other means known to a person skilled in the art. As well these means disclosed in this application can easily be part of a kit comprising probes, primers, oligonucleotides and any reagents required in the methodologies for detecting mutations that may cause glaucoma. These mutation analyses are useful for screening individuals at risk for glaucoma. Such individuals may have a family history of glaucoma, and, identifying individuals carrying a mutation in glaucoma gene would permit early treatment that may obviate or minimise the progression of the disease.

The invention and the applications thereof will be made obvious with the foregoing disclosure.

DEFINITIONS AND TECHNOLOGICAL BACKGROUND

Nucleotide sequences are presented herein by single strand, in the 5' to 3' direction, from left to right, using the one letter nucleotide symbols as commonly used in the art and in accordance with the recommendations of the IUPAC-IUB Biochemical Nomenclature Commission.

The present description refers to a number of routinely used recombinant DNA (rDNA) technology terms. Nevertheless, definitions of selected examples of such rDNA terms are provided for clarity and consistency.

As used herein, "isolated nucleic acid molecule", refers to a polymer of nucleotides. Non-limiting examples thereof include DNA and RNA molecules purified from their natural environment.

The term "recombinant DNA" as known in the art refers to a DNA molecule resulting from the joining of DNA segments. This is often referred to as genetic engineering.

The term "DNA segment", is used herein, to refer to a DNA molecule comprising a linear stretch or sequence of nucleotides. This sequence when read in accordance with the genetic code, can encode a linear stretch or sequence of amino acids which can be referred to as a polypeptide, protein, protein fragment and the like.

The terminology "amplification pair" or "primer pair" refers herein to a pair of oligonucleotides (oligos) of the present invention, which are selected to be used together in amplifying a selected

nucleic acid sequence by one of a number of types of amplification processes, preferably a polymerase chain reaction. Other types of amplification processes include ligase chain reaction, strand displacement amplification, or nucleic acid sequence-based amplification, as explained in greater detail below. As commonly known in the art, the oligos are designed to bind to a complementary sequence under selected conditions.

The nucleic acid (i.e. DNA or RNA) for practising the present invention may be obtained according to well known methods.

Oligonucleotide probes or primers of the present invention may be of any suitable length, depending on the particular assay format and the particular needs and targeted genomes employed. In general, the oligonucleotide probes or primers are at least 10 nucleotides in length, preferably between 15 and 24 nucleotides, and they may be adapted to be especially suited to a chosen nucleic acid amplification system. As commonly known in the art, the oligonucleotide probes and primers can be designed by taking into consideration the melting point of hydrizidation thereof with its targeted sequence (in Sambrook et al., 1989, Molecular Cloning - A Laboratory Manual, 2nd Edition, CSH Laboratories; Ausubel et al., 1989, in Current Protocols in Molecular Biology, John Wiley & Sons Inc., N.Y.).

"Nucleic acid hybridization" refers generally to the hybridization of two single-stranded nucleic acid molecules having complementary base sequences, which under appropriate conditions will form a thermodynamically favored double-stranded structure. Examples of hybridization conditions can be found in the two laboratory manuals referred above (Sambrook et al., 1989, *supra* and Ausubel et al., 1989

supra) and are commonly known in the art. In the case of a hybridization to a nitrocellulose filter, as for example in the well known Southern blotting procedure, a nitrocellulose filter can be incubated overnight at 65°C with a labeled probe in a solution containing 50% formamide, high salt (5 x SSC or 5 x SSPE), 5 x Denhardt's solution, 1% SDS, and 100 µg/ml denatured carried DNA (i.e. salmon sperm DNA). The non-specifically binding probe can then be washed off the filter by several washes in 0.2 x SSC/0.1% SDS at a temperature which is selected in view of the desired stringency: room temperature (low stringency), 42°C (moderate stringency) or 65°C (high stringency). The selected temperature is based on the melting temperature (Tm) of the DNA hybrid. Of course, RNA-DNA hybrids can also be formed and detected. In such cases, the conditions of hybridization and washing can be adapted according to well known methods by the person of ordinary skill. High stringency conditions will be preferably used (Sambrook et al.,1989, supra).

Probes of the invention can be utilized with naturally occurring sugar-phosphate backbones as well as modified backbones including phosphorothioates, dithionates, alkyl phosphonates and α-nucleotides and the like. Modified sugar-phosphate backbones are generally taught by Miller, 1988, Ann. Reports Med. Chem. 23:295 and Moran et al., 1987, Nucleic acid molecule. Acids Res., 14:5019. Probes of the invention can be constructed of either ribonucleic acid (RNA) or deoxyribonucleic acid (DNA), and preferably of DNA.

The types of detection methods in which probes can be used include Southern blots (DNA detection), dot or slot blots (DNA, RNA), and Northern blots (RNA detection). Although less prepared,

labelled proteins could also be used to detect a particular nucleic acid sequence to which it binds. Other detection methods include kits containing probes on a dipstick setup and the like.

Although the present invention is not specifically dependent on the use of a label for the detection of a particular nucleic acid sequence, such a label might be beneficial, by increasing the sensitivity of the detection. Furthermore, it enables automation. Probes can be labelled according to numerous well known methods (Sambrook et al., 1989, supra). Non-limiting examples of labels include ^{3}H, ^{14}C, ^{32}P, and ^{35}S. Non-limiting examples of detectable markers include ligands, fluorophores, chemiluminescent agents, enzymes, and antibodies. Other detectable markers for use with probes, which can enable an increase in sensitivity of the method of the invention, include biotin and radionucleotides. It will become evident to the person of ordinary skill that the choice of a particular label dictates the manner in which it is bound to the probe.

As commonly known, radioactive nucleotides can be incorporated into probes of the invention by several methods. Non-limiting examples thereof include kinasing the 5' ends of the probes using gamma ^{32}P ATP and polynucleotide kinase, using the Klenow fragment of Pol I of *E. coli* in the presence of radioactive dNTP (i.e. uniformly labelled DNA probe using random oligonucleotide primers in low-melt gels), using the SP6/T7 system to transcribe a DNA segment in the presence of one or more radioactive NTP, and the like.

As used herein, "oligonucleotides" or "oligos" define a molecule having two or more nucleotides (ribo or deoxyribonucleotides). The size of the oligo will be dictated by the particular situation and

ultimately by the particular use thereof, and adapted accordingly by the person of ordinary skill. An oligonucleotide can be synthetised chemically or derived by cloning according to well known methods.

As used herein, a "primer" defines an oligonucleotide which is capable of annealing to a target sequence, thereby creating a double stranded region which can serve as an initiation point for DNA synthesis under suitable conditions.

Amplification of a selected, or target, nucleic acid sequence may be carried out by a number of suitable methods. See generally Kwoh et al., 1990, (Am. Biotechnol. Lab. 8:14-25). Numerous amplification techniques have been described and can be readily adapted to suit the particular needs of a person of ordinary skill. Non-limiting examples of amplification techniques include polymerase chain reaction (PCR), ligase chain reaction (LCR), strand displacement amplification (SDA), transcription-based amplification, the Qβ replicase system and NASBA (Kwoh et al., 1989, Proc. Natl. Acad. Sci. USA 86, 1173-1177; Lizardi et al., 1988, BioTechnology 6:1197-1202; Malek et al., 1994, Methods Mol. Biol., 28:253-260; and Sambrook et al., 1989, *supra*). Preferably, amplification will be carried out using PCR.

Polymerase chain reaction (PCR) is carried out in accordance with known techniques. See, e.g., U.S. Pat. Nos. 4,683,195; 4,683,202; 4,800,159; and 4,965,188 (the disclosures of all three U.S. Patent are incorporated herein by reference). In general, PCR involves, a treatment of a nucleic acid sample (e.g., in the presence of a heat stable DNA polymerase) under hybridizing conditions, with one oligonucleotide primer for each strand of the specific sequence to be detected. An extension product of each primer which is synthesized is

complementary to each of the two nucleic acid strands, with the primers sufficiently complementary to each strand of the specific sequence to hybridize therewith. The extension product synthesized from each primer can also serve as a template for further synthesis of extension products using the same primers. Following a sufficient number of rounds of synthesis of extension products, the sample is analysed to assess whether the sequence or sequences to be detected are present. Detection of the amplified sequence may be carried out by visualization following EtBr staining of the DNA following gel electrophoresis, or using a detectable label in accordance with known techniques, and the like. For a review on PCR techniques (see PCR Protocols, A Guide to Methods and Amplifications, Michael et al., Eds, Acad. Press, 1990).

Ligase chain reaction (LCR) is carried out in accordance with known techniques (Weiss, 1991, Science 254:1292). Adaptation of the protocol to meet the desired needs can be carried out by a person of ordinary skill. Strand displacement amplification (SDA) is also carried out in accordance with known techniques or adaptations thereof to meet the particular needs (Walker et al., 1992, Proc. Natl. Acad. Sci. USA 89:392-396; and ibid., 1992, Nucleic Acids Res. 20:1691-1696).

As used herein, the term "gene" is well known in the art and relates to a nucleic acid sequence defining a single protein or polypeptide. A "structural gene" defines a DNA sequence which is transcribed into RNA and translated into a protein having a specific amino acid sequence thereby giving rise the a specific polypeptide or protein. It will be readily recognized by the person of ordinary skill, that the nucleic acid sequences of the present invention can be incorporated

into anyone of numerous established kit formats which are well known in the art.

The term "vector" is commonly known in the art and defines a plasmid DNA, phage DNA, viral DNA and the like, which can serve as a DNA vehicle into which DNA of the present invention can be cloned. Numerous types of vectors exist and are well known in the art.

The term "expression" defines the process by which a structural gene is transcribed into mRNA (transcription), the mRNA is then being translated (translation) into one polypeptide (or protein) or more.

The terminology "expression vector" defines a vector or vehicle, as described above, but designed to enable the expression of an inserted sequence following transformation into a host. The cloned gene (inserted sequence) is usually placed under the control of control element sequences such as promoter sequences. The placing of a cloned gene under such control sequences is often referred to as being "operably linked" to control elements or sequences.

Expression control sequences will vary depending on whether the vector is designed to express the operably linked gene in a prokaryotic or eukaryotic host or both (shuttle vectors) and can additionally contain transcriptional elements such as enhancer elements, termination sequences, tissue-specificity elements, and/or translational initiation and termination sites.

As used herein, the designation "functional derivative" denotes, in the context of a functional derivative of a sequence, whether nucleic acid or amino acid sequence, a molecul that retains a biological activity (either functional or structural) that is substantially similar to that

of the original sequence. This functional derivative or equivalent may be a natural derivative or may be prepared synthetically. Such derivatives include amino acid sequences having substitutions, deletions, or additions of one or more amino acids, provided that the biological activity of the protein is conserved. The same applies to derivatives of nucleic acid sequences which can have substitutions, deletions, or additions of one or more nucleotides, provided that the biological activity of the sequence is generally maintained. When relating to a protein sequence, the substituting amino acid has chemico-physical properties which are similar to that of the substituted amino acid. The similar chemico-physical properties include, similarities in charge, bulkiness, hydrophobicity, hydrophylicity and the like. The term "functional derivatives" is intended to include "fragments", "segments", "variants", "analogs" or "chemical derivatives" of the subject matter of the present invention.

Thus, the term "variant" refers herein to a protein or nucleic acid molecule which is substantially similar in structure and biological activity to the protein or nucleic acid of the present invention.

The functional derivatives of the present invention can be synthesized chemically or produced through recombinant DNA technology. All these methods are well known in the art.

As used herein, "chemical derivatives" is meant to cover additional chemical moieties not normally part of the subject matter of the invention. Such moieties could affect the physico-chemical characteristic of the derivative (i.e. solubility, absorption, half life and the like, decrease of toxicity). Such moieties are exemplified in R mington's Pharmaceutical Sciences (1980). Methods of coupling thes chemical-physical moieties to a polypeptide are well known in the art.

The term "allele" defines an alternative form of a gene which occupies a given locus on a chromosome.

As commonly known, a "mutation" is a detectable change in the genetic material which can be transmitted to a daughter cell. As well known, a mutation can be, for example, a detectable change in one or more deoxyribonucleotide. For example, nucleotides can be added, deleted, substituted for, inverted, or transposed to a new position. Spontaneous mutations and experimentally induced mutations exist. The result of a mutations of nucleic acid molecule is a mutant nucleic acid molecule. A mutant polypeptide can be encoded from this mutant nucleic acid molecule.

As used herein, the term "purified" refers to a molecule having been separated from a cellular component. Thus, for example, a "purified protein" has been purified to a level not found in nature. A "substantially pure" molecule is a molecule that is lacking in all other cellular components.

The term "autosome" defines any chromosome other that the sex chromosomes, X and Y.

The term "dominant" refers to an allele that determines the phenotype displayed in a heterozygote with another (recessive) allele.

The terminology "transgenic animal" defines an animal that has had its germ line genetically modified to give rise to a progeny animal that is different from the parental type and carrying the modification in its germ line.

"Single Strand Conformational Polymorphism (SSCP)" refers to a method for detecting the presence of a bas pair change in an

amplified DNA fragment. The method involves denaturing the double stranded amplified DNA and comparing the band pattern in a known non-mutant fragment to that of an unknown fragment. A shift in the band pattern is indicative of a base pair change.

The designation "gene therapy" defines an attempt to treat disease by genetic modification of the cells of a patient.

"Allele Specific Oligonucleotide (ASO)" are designed to detect known and identified base pair change by designing oligonucleotides that are specific to the DNA fragment with and without the base change. These oligonucleotides are used as probes in hybridisation protocols under stringent conditions. Differences in the hybridization patterns is indicative of the presence or absence of the base change.

"Artificially Created Restriction Site (ACRS)" refers to a method for detection a known base change in a DNA sequence. It involves the designing of a primer that may either create or obviate a restriction site in the vicinity of known base change, such that the restriction endonuclease used can have a different digestion pattern for the changed and unchanged base.

BRIEF DESCRIPTION OF THE DRAWINGS

Having thus generally described the invention, reference will now be made to the accompanying drawings, showing by way of illustration a preferred embodiment thereof, and in which:

Figure 1 shows the sequence that encodes the wild-type *GLC1A/TIGR* cDNA sequence.

Figure 2 shows the characterization of a carrier homozygous for the Lys423Glu *TIGR* mutation. *a*, Structure of the *TIGR* encoded protein. The leucine zipper domain (amino acids 117-166) is shown within the N-terminal half of the protein. The Lys423Glu mutation is depicted by an open circle in the olfactomedin homology domain represented by a striped boxe within the C-terminal half of the protein. Amino acids comparison between human *TIGR* protein (amino acids 415-437), human neuronal olfactomedin, rat and bullfrog neuronal olfactomedin-related proteins (GeneBank accession U79299, U03417, L13595, respectively) and, C. elegans F11c3.2 protein (GeneBank accession Z81499) is represented. Identical amino acids are shaded in black, conserved amino acids are further boxed by white squares. The codon numbers correspond to those of the *TIGR* protein, b, Identification of an homozygous carrier of the Lys423Glu *TIGR* mutation. Direct sequencing of genomic DNA revealed that persons VI-3 and VI-9 were, respectively, heterozygotic and homozygotic carriers of the Lys423Glu *TIGR* mutation. The arrows indicate the A to G transition. Person VI-2 carried two wild-type *TIGR* alleles.

Figure 3 shows the amplification refractory mutation system (ARMS) as a method to type specific alleles at a polymorphic locus. In the present invention, this method, ARMS, was used for detecting a specific pathogenic mutation. The allele-specific oligonucleotide primers were designed to discriminate between two target DNA sequences (wild-type (normal) versus pathogenic) that differed by a single nucleotide in the region of interest (either one of the two mutations). Designed primers that differed at the xtreme 3' terminus were synthetised. This was done because the DNA synthesis step in the

PCR reaction is crucially dependent on correct base pairing at the 3' end. The primers that were designed are differing in their 3' ends and can therefore specifically amplify the DNA fragment of interest, either normal or mutated. This figure is a pictorial representation of ARMS for the adenine to guanine transition at nucleotide 1267. The amplification strategy is demonstrated for the wild-type or non-mutant allele and the mutant allele.

Figure 4 shows the phenotypic status and segregation analyses of the *GLC1A* disease haplotype and Lys423Glu in family GV-510. All living individuals were investigated for glaucoma, genotyped with microsatellite markers spanning the *GLC1A* locus and tested for the presence of the Lys423Glu *TIGR* mutation using ARMS. Selected AFM markers with their corresponding GDB number, number of alleles observed for each marker in pedigree GV-001 and sizes of the allele associated with the *GLC1A* disease haplotype are represented on top. The position of the *TIGR* gene is indicated relative to genetic markers. Sex-averaged recombination distances, depicted between marker loci in centiMorgans, were not drawn to scale. Glaucoma patients are depicted by solid black symbols, unaffected individuals by open symbols, and deceased subjects reported as blind by at least two independent family members by a black quadrant in the upper left corner of their respective symbols. OHT persons are represented by open symbols containing a central solid dot. Present ages of normal and OHT patients as well as ages of affected carriers at time of diagnosis are depicted above their respective symbols. A solid black box indicates the common *GLC1A* disease haplotype. The right side of each phased haplotype indicates the haplotype inherited from the father; the left side indicates the haplotype

inherited from the mother. An asterisk in the genotype of person VII-5 represents a microsatellite mutation at locus D1S2790. Person VII-5 also inherited a paternal recombination between loci D1S2815 and D1S2790. Results of the ARMS tests are depicted below each subject's genotype; W, ARMS test performed using the wild-type primers; M, ARMS test performed using the Lys423Glu mutant primers. The internal control PCR product is shown. Persons VI-2, VI-5, VI-6, VI-10 and VI-12 carried the wild-type allele on both chromosomes 1. Persons VI-1, VI-7, VI-9 and VI-11 are wild-type negative and mutant positive, therefore, homozygous for the Lys423Glu mutation. All other individuals are both wild-type positive and mutant positive, therefore, heterozygotes for the mutation.

Figure 5 shows the characterization of carriers for the HIS366Gln and Gln368Stop *TIGR* mutations. *a*, Structure of the *TIGR* encoded protein. The leucine zipper domain (amino acids 117-166) is shown within the N-terminal half of the protein. The His366Gln mutation is depicted by a black circle in the olfactomedin homology domain represented by a striped boxe within the C-terminal half of the protein. The Gln368Stop mutation is depicted by a stop codon in the olfactomedin homology domain. The codon numbers correspond to those of the *TIGR* protein. *b*, Identification of carriers for the His366Gln and Gln368Stop *TIGR* mutations. Direct sequencing of genomic DNA revealed that persons CT-003 and LA-002 were, respectively, heterozygotic carriers of the Gln368Stop and His366Gln *TIGR* mutations. The arrows indicate the C to T transition or C to G tranversion.

Other objects, advantages and features of the present invention will become more apparent upon reading of the following non-restrictive description of preferred embodiments with reference to the

accompanying drawing which is exemplary and should not be interpreted as limiting the scope of the present invention.

DESCRIPTION OF THE PREFERRED EMBODIMENT

At its broadest, the invention comprises novel mutations in the *TIGR* gene, a quick method for an easy detection of identified mutations and the teachings for the first time of mutant homozygote being phenotypically normal in an autosomally dominant inherited disease.

The present invention is illustrated in further detail by the following non-limiting examples.

EXAMPLE 1

Pedigrees and ophthalmologic assessments

1.1 Pedigree reconstitution

The pedigree genealogy was reconstituted using the registers compiled from the Catholic parish records, which systematically list births, marriages, and deaths of 98% of the Quebec population. Validation of the family tree and new data on recent births were obtained through interviews with key family members. The Archives Nationales du Quebec, the Quebec Civil register, and the Institut de recherche sur l'étude des populations (IREP) data base (Bouchard et al., 1991, Histoire d'un génome. Population et génétique dans l'est du Quebec, Presses de l'Université Laval, Sillery, Quebec, pp 607) were also consulted.

1.2 Ophthalmologic investigations

All subjects, affected or not, gave informed consent before entering the study. Clinical assessments comprised complete ophthalmologic evaluation, including best corrected visual acuity; optic disk examination; slit-lamp biomicroscopy; applanation tonometry; gonioscopy; and visual-field evaluation. Three criteria were required for primary open-angle glaucoma (POAG) diagnosis: a) intraocular pressures above 22 mm Hg in both eyes, b) characteristic optic disk damage and/or visual field impairment, and c) grade III or IV (open-angle) gonioscopy. In the absence of optic disk damage or visual-field alteration, subjects with intraocular pressures above 22 mm Hg in both eyes and grade III or IV gonioscopy were diagnosed with ocular hypertension (OHT). Members of the families were considered normal when they presented normal optic disks and showed highest intraocular pressures ever recorded at 22 mm Hg or less. Persons with other forms of glaucomas, including grade 0 (closed angle); grade I or II (narrow-angle); congenital; and secondary glaucomas, or with other nonglaucomatous ocular disorders were considered unaffected. Blindness in deceased ancestors was confirmed by at least two independent sources.

EXAMPLE 2

2.1 Source of DNA

Blood samples were obtained from direct descendants of the founder as well as spouses of affect d patients with children; from each, 20 ml of blood was drawn by venipuncture in heparinized tubes. On additional 10 ml blood sample was drawn from each subject to establish

lymphoblastoid cell lines using the method of Anderson et al. (1984, In Vitro, 20:856-858).

2.2 Isolation of DNA

DNA was extracted from whole blood using the guanidine hydrochloride-proteinase K method developed by Jeanpierre (1987, Nucl. Acids. Res. 15:9611-9611).

2.3 Genotyping procedures

To accelerate genotyping, we used a protocol similar to the procedure of Vignal et al. (1993, Methods in molecular genetics, Academic Press, 1:211-221) which was derived from the multiplex sequencing technique of Church and Kieffer-Higgins (1988 Science 240:185-188). Briefly, polymerase chain reactions (PCR) were performed in a total volume of 50 µl containing 100 ng of genomic DNA, 50 pmol of each primer, 125 mM dNTPs, 50 mM KCl, 10 mM Tris (pH 9), 1.5 mM $MgCl_2$, 0.01% gelatin, 0.1% Triton X-100, and 1 U Taq polymerase (Perkin-Elmer-Cetus). Amplifications were carried out using a "hot-start" procedure. Taq polymerase was added after a 5-min denaturation step at 96°C. Samples were then processed through 35 cycles of denaturation (94°C for 40 s) and annealing (55°C for 30 s), followed by one last step of elongation (2 min at 72° C). Usually, three amplification products synthesized with separate primer sets on identical DNA samples were coprecipitated and comigrated in a single lane of 6% polyacrylamide denaturing gels. Separated products were then transferred onto Hybond N^+ nylon membranes (Amersham), hybridized with a $(CA)_{20}$ oligomer 3' labeled with Digoxigenin-11-ddUTP, and detected by ch miluminescence

using the DIG system (Boehringer-Mannheim) with Kodak XAR-5 films. Genotypes were scored relative to reference alleles of the mother of the CEPH family 1347 (individual 134702). Genotyping was repeated upon detection of incompatibilities or recombination events.

2.4 Selection of microsatellite markers

In Figure 4, the markers used for haplotype analyses are shown. With the exception of two markers (AFMGLC21 and AFMGLC22), all AFM (Généthon) markers reported above were described in Dib et al. (1996, supra). For AFMGLC21, the sequences were primer a: GATCTCTTATCAGTCAGGCA, and primer m: TTTCTAAGGCTGAATAATATTCG. For AFMGLC22, the sequences were primer a: TTAACTCACCACTCCCTGCC, and primer m: AATTATGGCCTTCGCCC. Assignment of the genetic location of these markers was established according to the method of Weissenbach et al. (1992, Nature, 359:795-801) and has been validated by construction of a 10-cM physical map (Clépet et al., 1996, Eur. J. Hum.Genet., 4:250-259).

2.5 Haplotype analysis

Haplotypes were analysed to phase the marker genotypes with the disease gene. The haplotype inherited by an affected child constituted the "disease" haplotype and was compared with the common disease haplotype inherited from the founder. The remaining three haplotypes were considered the "normal" haplotypes.

EXAMPLE 3

Discovery of the phenotypic normal-homozygote mutant

3.1 Phenotypic normal-homozygote mutant (Figure 4)

Phenotypic status and segregation analyses of the *GLC1A* disease haplotype and Lys423Glu *TIGR* mutation in family GV-510. All living individuals were investigated for glaucoma, genotyped with microsatellite markers spanning the *GLC1A* locus and tested for the presence of the Lys423Glu *TIGR* mutation using ARMS. Selected AFM markers with their corresponding GDB number, number of alleles observed for each marker in pedigree GV-001 and sizes of the allele associated with the *GLC1A* disease haplotype are represented on top. The position of the *TIGR* gene is indicated relative to genetic markers. Sex-averaged recombination distances, depicted between marker loci in centiMorgans, were not drawn to scale. Glaucoma patients are depicted by solid black symbols, unaffected individuals by open symbols, and deceased subjects reported as blind by at least two independent family members by a black quadrant in the upper left corner of their respective symbols. OHT persons are represented by open symbols containing a central solid dot. Present ages of normal and OHT patients as well as ages of affected carriers at time of diagnosis are depicted above their respective symbols. A solid black box indicates the common *GLC1A* disease haplotype. The right side of each phased haplotype indicates the haplotype inherited from the father; the left side indicates the haplotype inherited from the mother. An asterisk in the genotype of person VII-5 represents a microsatellite mutation at locus D1S2790. Person VII-5 also inherited a paternal recombination

between loci D1S2815 and D1S2790. Results of the ARMS tests are depicted below each subject's genotype; W, ARMS test performed using the wild-type primers; M, ARMS test performed using the Lys423Glu mutant primers. The internal control PCR product is shown. Persons VI-2, VI-5, VI-6, VI-10 and VI-12 carried the wild-type allele on both chromosomes 1. Persons VI-1, VI-7, VI-9 and VI-11 are wild-type negative and mutant positive, therefore, homozygous for the Lys423Glu mutation. All other individuals are both wild-type positive and mutant positive, therefore, heterozygotes for the mutation.

3.2 Initial screening for mutations

To obtain a wild-type *TIGR* cDNA, RT-PCR was performed using the Superscript RT protocol (Gibco/BRL), 500 ng of oligo-dT and 10 µg of total RNA isolated from a pool of trabecular meshwork tissue dissected from 10 pairs of human eyes. To obtain the mutated *TIGR* cDNA, the same protocol was followed using 10 µg of total RNA isolated from homozygote VI-9 immortalized lymphoblasts. One to 3 µl of first strand cDNA synthesis was amplified with primers 41F: AGAGCTTTCCAGAGGAAGCC, and 1731R: GGTCTACGCCCTCAGACTAC, before a second round of PCR with internal primers 31F: AGAGACAGCAGCACCCAACG, and 21R: TCTGCCATTGCCTGTACAGC. PCR products were directly cloned into the pCRII vector using the TA cloning kit (InVitrogen) according to the manufacturer's protocol. Cloned products were sequenced using the T7 sequencing kit (Pharmacia).

3.3 Sequencing

To confirm mutations, genomic DNA sequencing was also performed on selected individuals by direct asymmetric PCR sequencing using modifications of the protocol described by Gyllensten et al. (1988, Proc. Natl. Acad. Sci., 85:7652-7656). The mutation was recognized by the approximately equal peak intensity of the bands on the autoradiogram. All sequencing was performed bidirectionally.

EXAMPLE 4

Two mutations including ARMS

4.1 ARMS test for the Lys423Glu mutation (Figure 3)

To test for the presence of the Lys423Glu mutation, we developed an amplification refractory mutation system (ARMS) exploiting procedures described by Little (1997, Current Protocols in human genetics, Eds. Dracopoli, N.C. et al., 9.8.1. - 9.8.12). Two complementary PCR reactions were conducted with the same substrate. The first reaction contained a forward primer specific for the wild-type allele, SEQ. NO. 3, *GLC1A*1313AA: TCGAACAAACCTGGGAGACAAACATCCGAA. The second reaction contained a forward primer specific for the Lys423Glu *TIGR* allele, SEQ. NO. 2, *GLC1A*1313GG: TCGAACAAACCTGGGAGACAAACATCCGGG. In each reaction, a common reverse primer, *GLC1A*1479R, was used; its sequence was:

SEQ. NO. 4 CAAAGAGCTTCTTCTCCAGGGGGTTGTAGT. Both reactions gave a 225 bp amplified fragment. To serve as internal control,

a second pair of primers that co-amplified a 438 bp fragment within *TIGR* exon 1 was added to the ARMS reaction. The forward *TIGR* exon 1 primer was: AGAGCTTTCCAGAGGAAGCC, the reverse *TIGR* exon 1 primer was TTGGGTTTCCAGCTGGTC. PCR was performed using standard protocols, annealing temperature was at 60°C. Amplification products were electrophoresed in 1,5% agarose gels before ethidium staining and scored by two independent observers.

4.2 ARMS test for the His366Gln mutation

To test for the presence of the His366Gln mutation, we developed an amplification refractory mutation system (ARMS) exploiting procedures described by Little (1997). Two complementary PCR reactions were conducted with the same substrate. The first reaction contained a forward primer specific for the wild-type allele, SEQ. NO. 6 *GLC1A*1098CT: GAGAAGGAAATCCCTGGAGCTGGCTACCTC. The second reaction contained a forward primer specific for the His366Gln *TIGR* allele, SEQ. NO. 5, *GLC1A*1098GT: GAGAAGGAAATCCCTGGAGCTGGCTACCTG. In each reaction, a common reverse primer, *GLC1A*1479R, was used; its sequence was:

SEQ. NO. 7, CAAAGAGCTTCTTCTCCAGGGGGTTGTAGT. Both reactions gave a 393 bp amplified fragment. To serve as internal control, a second pair of primers that co-amplified a 438 bp fragment within *TIGR* exon 1 was added to the ARMS reaction. The forward *TIGR* exon 1 primer was: AGAGCTTTCCAGAGGAAGCC, the reverse *TIGR* exon 1 primer was TTGGGTTTCCAGCTGGTC. PCR was performed using standard protocols, annealing temperature was at 60°C. Amplification

products were electrophoresed in 1.5% agarose gels before ethidium staining and scored by two independent observers.

Although the present invention has been described hereinabove by way of preferred embodiments thereof, it can be modified, without departing from the spirit and nature of the subject invention as defined in the appended claims.

WHAT IS CLAIMED IS:

1. An isolated DNA comprising the nucleotide sequence defined in SEQ. ID. NO.: 1, wherein the nucleotide located at position 1267 is a guanidine residue in lieu of an adenine residue , said guanidine residue being a specific nucleotide of a mutant allele of the *TIGR* gene.

2. An isolated DNA comprising the nucleotide sequence defined in SEQ. ID. NO.: 1, wherein the nucleotide located at position 1096 is a guanidine residue in lieu of a cytosine residue , said guanidine residue being a specific nucleotide of a mutant allele of the *TIGR* gene.

3. An oligonucleotide comprising at least 10 nucleotides of SEQ. ID. NO.: 1, said oligonucleotide ending at its 3' end with said specific nucleotide , as defined in claim 1, or a complementary sequence thereof.

4. An oligonucleotide comprising at least 10 nucleotides of SEQ. ID. NO.: 1, said oligonucleotide ending at its 3' end with a non mutant nucleotide corresponding to said specific nucleotide, as defined in claim 1, or a complementary sequence thereof.

5. An oligonucleotide as defined in claim 3, which is incapabl of priming a polymerase priming extension when annealed to a non mutant allel .

6. An oligonucleotide as defined in claim 4, which is incapable of priming a polymerase priming extension when annealed to said mutant allele.

7. An oligonucleotide as defined in claim 5, which has the nucleotide sequence of SEQ. ID. NO.: 2.

8. An oligonucleotide as defined in claim 6, which has the nucleotide sequence of SEQ. ID. NO.: 3.

9. An oligonucleotide comprising at least 10 nucleotides of SEQ. ID. NO.: 1, said oligonucleotide having a nucleotide sequence shared by said mutant allele and a non mutant allele, as defined in claim 1, and a complementary sequence thereof.

10. An oligonucleotide as defined in claim 9, which has the nucleotide sequence of SEQ. ID. NO.: 4.

11. An oligonucleotide comprising at least 10 nucleotides of SEQ. ID. NO.: 1, said oligonucleotide ending at its 3' end with said specific nucleotide , as defined in claim 2, or a complementary sequence thereof.

12. An oligonucleotide comprising at least 10 nucleotides of SEQ. ID. NO.: 1, said oligonucleotide ending at its 3' end

with a non mutant nucleotide corresponding to said specific nucleotide, as defined in claim 2, or a complementary sequence thereof.

13. An oligonucleotide as defined in claim 11, which is incapable of priming a polymerase priming extension when annealed to a non mutant allele.

14. An oligonucleotide as defined in claim 12, which is incapable of priming a polymerase priming extension when annealed to said mutant allele.

15. An oligonucleotide as defined in claim 13 ,which has the nucleotide sequence of SEQ. ID. NO.: 5.

16. An oligonucleotide as defined in claim 14, which has the nucleotide sequence of SEQ. ID. NO.: 6.

17. An oligonucleotide comprising at least 10 nucleotides of SEQ. ID. NO.: 1, said oligonucleotide having a nucleotide sequence shared by said mutant allele and a non mutant allele, as defined in claim 2, and a complementary sequence thereof.

18. An oligonucleotide as defined in claim 17, which has the nucleotide sequence of SEQ. ID. NO.: 4.

19. A method for detecting a mutant allele of the *TIGR* gene which comprises the steps of contacting a DNA sample taken from an individual with an oligonucleotide as defined in claims 3, 5 or 7 and with an oligonucleotide as defined in claim 9 or 10; obtaining an amplified product in an amplification reaction; and detecting said amplification product as an indication of the presence of said mutant allele.

20. A method for detecting a mutant allele of the *TIGR* gene which comprises the steps of contacting a DNA sample taken from an individual, with an oligonucleotide as defined in claims 11,13 or 15 and with an oligonucleotide as defined in claim 17 or 18; obtaining an amplified product in an amplification reaction; and detecting said amplification product as an indication of the presence of said mutant allele.

21. A method for detecting a non-mutant allele of the *TIGR* gene which comprises the steps of contacting a DNA sample taken from an individual, with an oligonucleotide as defined in claim 4, 6 or 8 and with an oligonucleotide as defined in claim 9 or 10; obtaining an amplified product in an amplification reaction; and detecting said amplification product as an indication of the presence of said non-mutant allele.

22. A method for detecting a non-mutant allel of the *TIGR* gene which comprises the steps of contacting a DNA sample taken from an individual, with an oligonucleotide as defined in claim 12, 14 or

16 and with an oligonucleotide as defined in claim 17 or 18; obtaining an amplified product in an amplification reaction; and detecting said amplification product as an indication of the presence of said non-mutant allele.

23. A kit for the detection of mutations in the *TIGR* gene comprising an oligonucleotide as defined in any one of claims 3, 5, 7, 11,13 and 15; an oligonucleotide as defined in any one of claims 4, 6, 8, 12, 14 and 16; and an oligonucleotide as defined in any one of claims 9, 10, 17 and 18; and suitable reagents required for obtaining amplified products in an amplification reaction.

24. The kit of claim 23, wherein amplification products are detectable.

25. A method for detecting in an individual the inheritance of two of said mutant alleles as defined in claim 1, said individual being homozygote for said mutant allele is phenotypically normal and said individual is capable of transmitting the said mutant allele to an offspring whereby said offspring is at risk for developing glaucoma, which comprises the steps of reproducing the methods of claims 19 and 21; a positive result obtained from the method of claim 19 and a negative result from the method of claim 21, being an indication that said individual is homozygote for said mutant allel .

ABSTRACT OF THE DISCLOSURE

The present invention discloses the discovery that in an autosomally inherited disease, a homozygote mutant is found to be phenotypically normal and the uses of such a knowledge thereof. The present invention has designed an easy and efficient means to detect mutations in the *GLC1A/TIGR* gene.

Wild-Type GLC1A/TIGR cDNA sequence

```
                    -36                              -10
                    agagct ttccagagga agcctcacca agcctctgca
+1
ATG AGG TTC TTC TGT GCA CGT TGC TGC AGC TTT GGG CCT GAG ATG
met arg phe phe cys ala arg cys cys ser phe gly pro glu met
+1

CCA GCT GTC CAG CTG CTG CTT CTG GCC TGC CTG GTG TGG GAT GTG
pro ala val gln leu leu leu leu ala cys leu val trp asp val

91
GGG GCC AGG ACA GCT CAG CTC AGG AAG GCC AAT GAC CAG AGT GGC
gly ala arg thr ala gln leu arg lys ala asn asp gln ser gly
31

CGA TGC CAG TAT ACC TTC AGT GTG GCC AGT CCC AAT GAA TCC AGC
arg cys gln tyr thr phe ser val ala ser pro asn glu ser ser

181
TGC CCA GAG CAG AGC CAG GCC ATG TCA GTC ATC CAT AAC TTA CAG
cys pro glu gln ser gln ala met ser val ile his asn leu gln
61

AGA GAC AGC AGC ACC CAA CGC TTA GAC CTG GAG GCC ACC AAA GCT
arg asp ser ser thr gln arg leu asp leu glu ala thr lys ala

271
CGA CTC AGC TCC CTG GAG AGC CTC CTC CAC CAA TTG ACC TTG GAC
arg leu ser ser leu glu ser leu leu his gln leu thr leu asp
91

CAG GCT GCC AGG CCC CAG GAG ACC CAG GAG GGG CTG CAG AGG GAG
gln ala ala arg pro gln glu thr gln glu gly leu gln arg glu
```

361
CTG GGC ACC CTG AGG CGG GAG CGG GAC CAG CTG GAA ACC CAA ACC
leu gly thr leu arg arg glu arg asp gln leu glu thr gln thr
121

AGA GAG TTG GAG ACT GCC TAC AGC AAC CTC CTC CGA GAC AAG TCA
arg glu leu glu thr ala tyr ser asn leu leu arg asp lys ser

451
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val leu glu glu glu lys lys arg leu arg gln glu asn glu asn
151

CTG GCC AGG AGG TTG GAA AGC AGC AGC CAG GAG GTA GCA AGG CTG
leu ala arg arg leu glu ser ser ser gln glu val ala arg leu

541
AGA AGG GGC CAG TGT CCC CAG ACC CGA GAC ACT GCT CGG GCT GTG
arg arg gly gln cys pro gln thr arg asp thr ala arg ala val
181

CCA CCA GGC TCC AGA GAA GTT TCT ACG TGG AAT TTG GAC ACT TTG
pro pro gly ser arg glu val ser thr trp asn leu asp thr leu

631
GCC TTC CAG GAA CTG AAG TCC GAG CTA ACT GAA GTT CCT GCT TCC
ala phe gln glu leu lys ser glu leu thr glu val pro ala ser
211

CGA ATT TTG AAG GAG AGC CCA TCT GGC TAT CTC AGG AGT GGA GAG
gly ile leu lys glu ser pro ser gly tyr leu arg ser gly glu

721
GGA GAC ACC GGA TGT GGA GAA CTA GTT TGG GTA GGA GAG CCT CTC
gly asp thr gly cys gly glu leu val trp val gly glu pro leu
241

ACG CTG AGA ACA GCA GAA ACA ATT ACT GGC AAG TAT GGT GTG TGG
thr leu arg thr ala glu thr ile thr gly lys tyr gly val trp

811
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met arg asp pro lys pro thr tyr pro tyr thr gln glu thr thr
271

TGG AGA ATC GAC ACA GTT GGC ACG GAT GTC CGC CAG GTT TTT GAG
trp arg ile asp thr val gly thr asp val arg phe val phe glu

901
TAT GAC CTC ATC AGC CAG TTT ATG CAG GGC TAC CCT TCT AAG GTT
tyr asp leu ile ser gln phe met gln gly tyr pro ser lys val
301

CAC ATA CTG CCT AGG CCA CTG GAA AGC ACG GGT GCT GTG GTG TAC
his ile leu pro arg pro leu glu ser thr gly ala val val tyr

991
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ser gly ser leu tyr phe gln gly ala glu ser arg thr val ile
331

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arg tyr glu leu asn thr glu thr val lys ala glu lys glu ile

1081
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pro gly ala gly tyr his gly gln phe pro tyr ser trp gly gly
361 366

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1171
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ile tyr ser thr asp glu ala lys gly ala ile val leu ser lys
391

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1261
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ile arg lys gln ser val ala asn ala phe ile ile cys gly thr
421 423

TTG TAC ACC GTC AGC AGC TAC ACC TCA GCA GAT GCT ACC GTC AAC
leu tyr thr val ser ser tyr thr ser ala asp ala thr val asn

1351
TTT GCT TAT GAC ACA GGC ACA GGT ATC AGC AAG ACC CTG ACC ATC
phe ala tyr asp thr gly thr gly ile ser lys thr leu thr ile
451

CCA TTC AAG AAC CGC TAT AAG TAC AGC AGC ATG ATT GAC TAC AAC
pro phe lys asn arg tyr lys tyr ser ser met ile asp tyr asn

1441
CCC CTG GAG AAG AAG CTC TTT GCC TGG GAC AAC TTG AAC ATG GTC
pro leu glu lys lys leu phe ala trp asp asn leu asn met val
481

1486
ACT TAT GAC ATC AAG CTC TCC AAG ATG TGA aaagcctcc aagctgtaca
thr tyr asp ile lys leu ser lys met STOP
496

1535
ggcaatggca gaaggagatg ctcagggctc ctgggggggag caggctgaag

1585
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1635
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1685
gtagtctgag ggcgtagacc atttcatata ataaatatcc tttatcttct

1735
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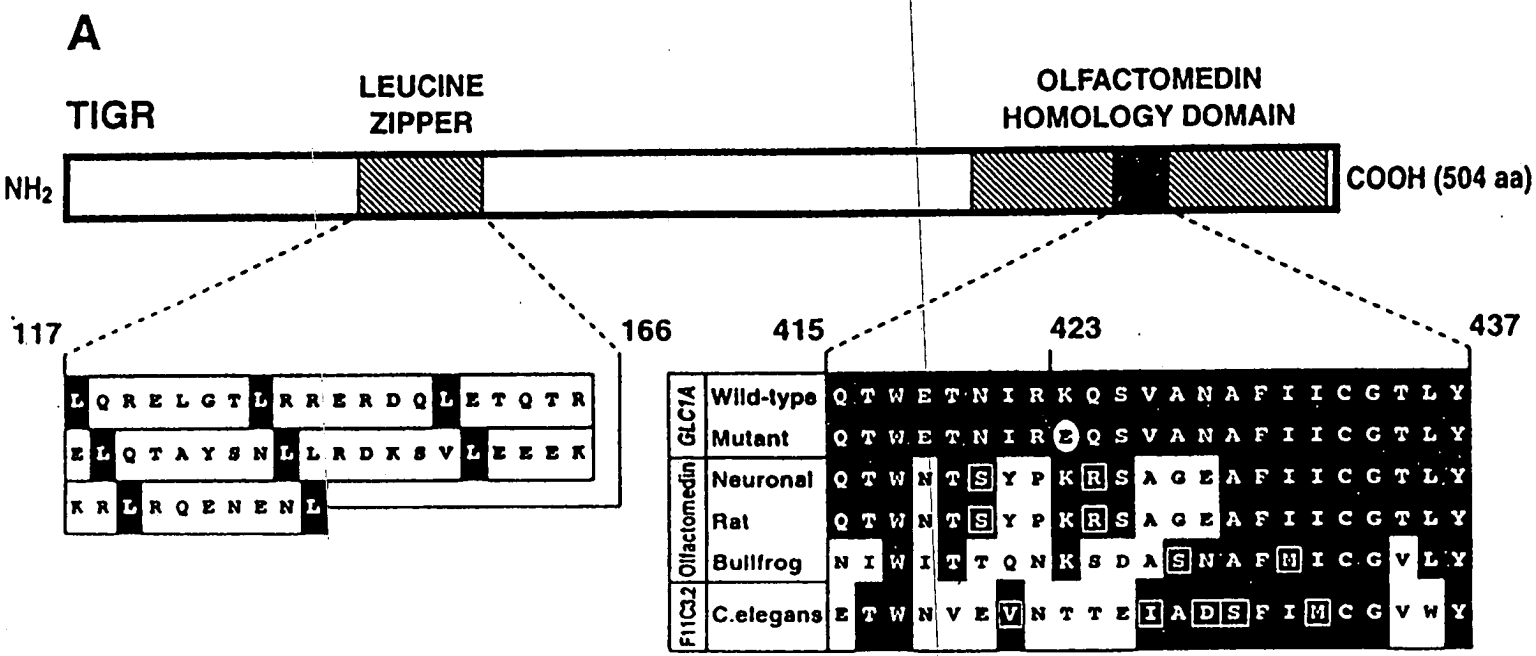
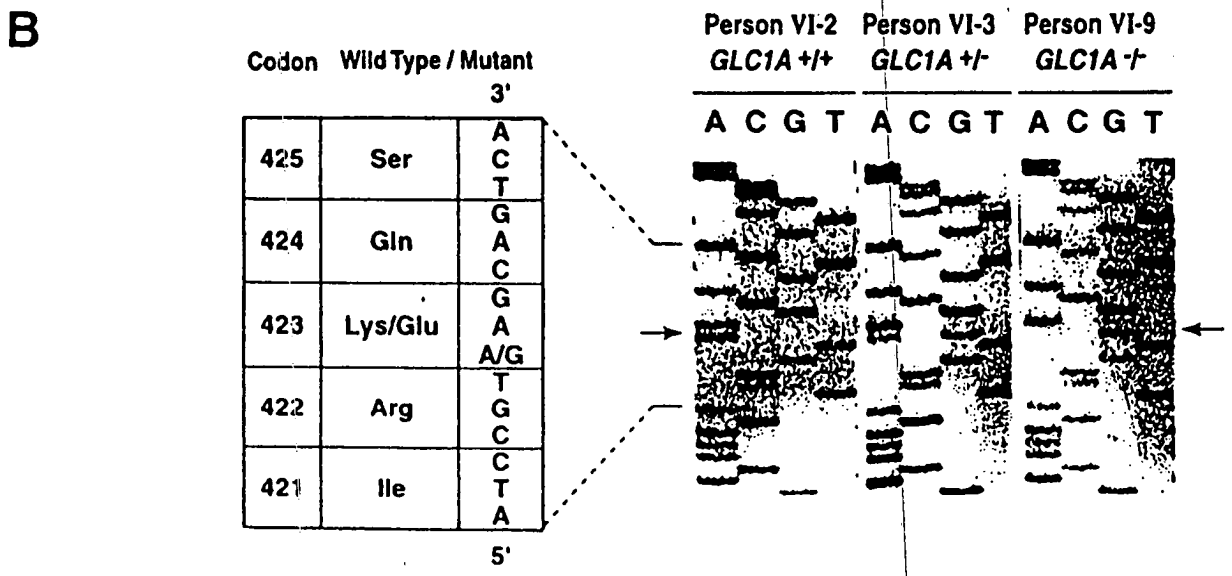


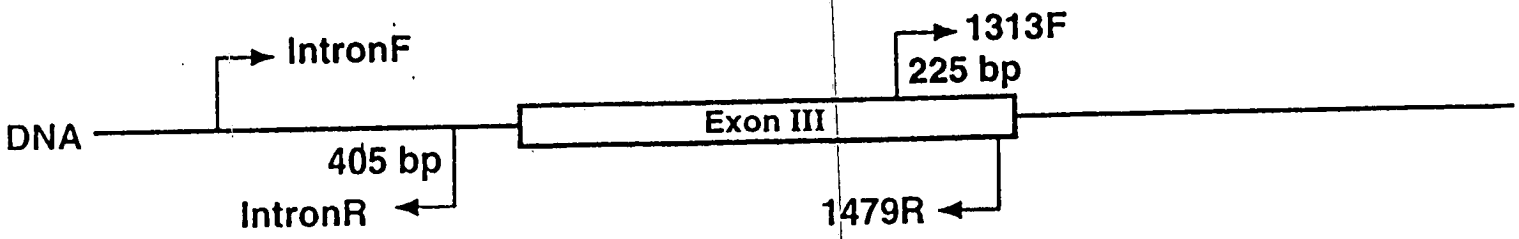


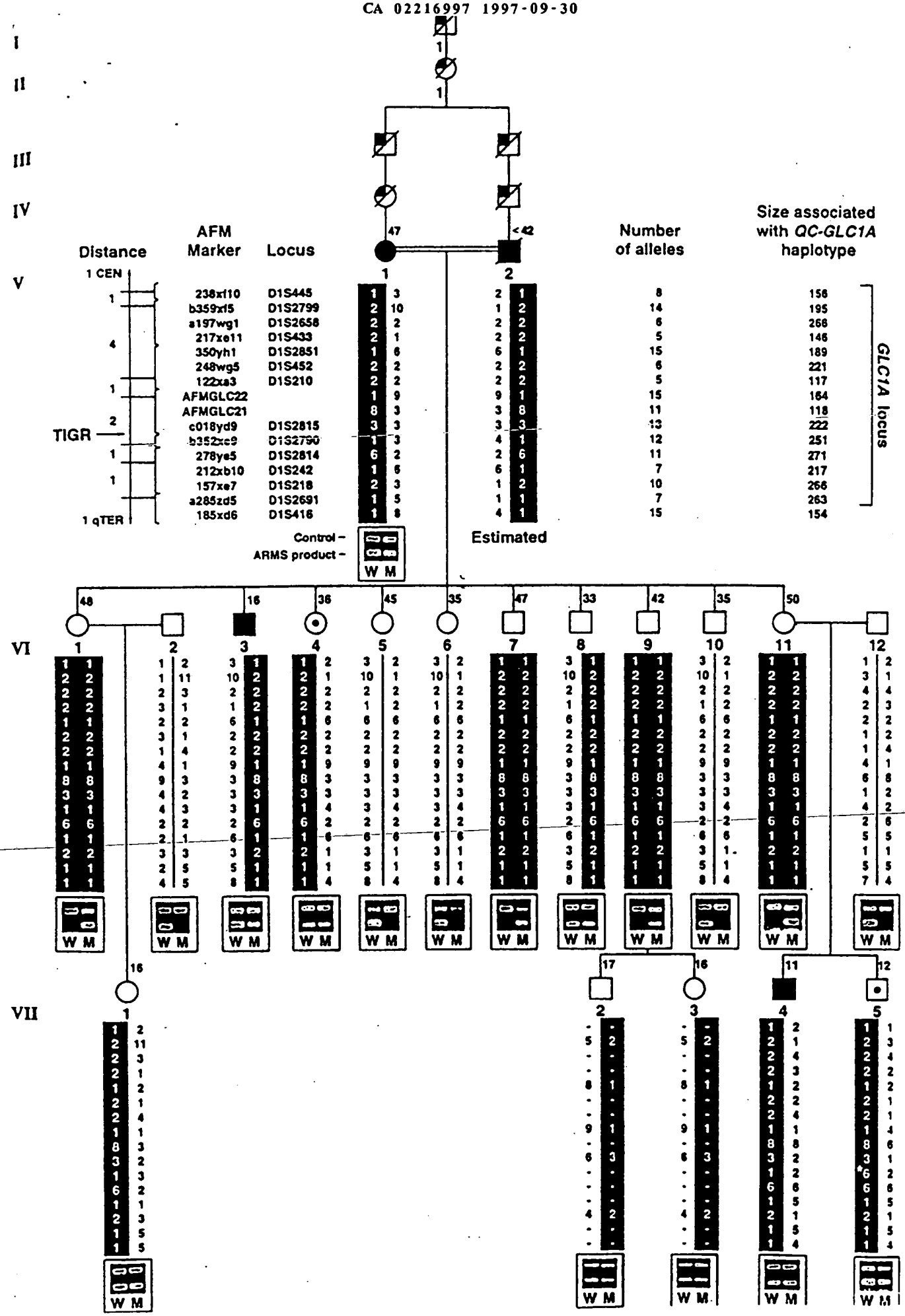
AMPLIFICATION REFRACTORY MUTATION SYSTEM

A

	Wild-type	Lys423Glu mutant
Wild-type Primer	A GCCTAC...5′ 5′...TGCTTACGGATGTTTGT...3′ 1313	A A GCCTAC...5′ 5′...TGCTCACGGATGTTTGT...3′ 1313
Mutant Primer	G G GCCTAC...5′ 5′...TGCTTACGGATGTTTGT...3′ 1313	G GCCTAC...5′ 5′...TGCTCACGGATGTTTGT...3′ 1313

B




I
II
III
IV
V
VI
VII
Distance
AFM Marker
Locus
Number of alleles
Size associated with QC-GLC1A haplotype
1 CEN
TIGR
1 qTER
238xf10 D1S445
b359xf5 D1S2799
a197wg1 D1S2658
217xe11 D1S433
350yh1 D1S2851
248wg5 D1S452
122xa3 D1S210
AFMGLC22
AFMGLC21
c018yd9 D1S2815
b352xc9 D1S2790
278ye5 D1S2814
212xb10 D1S242
157xe7 D1S218
a285zd5 D1S2691
185xd6 D1S416
GLC1A locus
Control –
ARMS product –
Estimated
W M